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3. Jarvis et al
Proc. Natl. Acad. Sci. 92(17):7996-8000, 1995
4. Jarvis et al
Infection and Immunity 64(11):4826-4829, 1996
5. Kenny et al
Proc. Natl. Acad. Sci. 92(17):7991-7995, 1995

Thank you

Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals to epithelial cells

(signal transduction/pathogenesis)

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Communicated by Harley W. Moon, U.S. Department of Agriculture, Ames, IA, May 2, 1995

ABSTRACT Enteropathogenic *Escherichia coli* (EPEC), a major cause of pediatric diarrhea, adheres to epithelial cells and activates host cell signal transduction pathways. We have identified five proteins that are secreted by EPEC and show that this secretion process is critical for triggering signal transduction events in epithelial cells. Protein secretion occurs via two pathways: one secretes a 110-kDa protein and the other mediates export of the four remaining proteins. Secretion of all five proteins was regulated by temperature and the *perA* locus, two factors which regulate expression of other known EPEC virulence factors. Amino-terminal sequence analysis of the secreted polypeptides identified one protein (37 kDa) as the product of the *eaeB* gene, a genetic locus previously shown to be necessary for signal transduction. A second protein (39 kDa) showed significant homology with glyceraldehyde-3-phosphate dehydrogenase, while the other three proteins (110, 40, and 25 kDa) were unique. The secreted proteins associated with epithelial cells, and EaeB became resistant to protease digestion upon association, suggesting that intimate interactions are required for transducing signals.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of acute and persistent infantile diarrhea (1) and remains a leading cause of infant death in developing countries. The mechanisms by which EPEC interacts with humans to cause disease do not seem to involve enterotoxins (1) but instead appear to be linked to bacterial adherence to intestinal epithelial cells. Morphological studies on both *in vivo* and *in vitro* infection systems have identified two distinguishable stages of adherence by EPEC. The first, localized adherence (2, 3), is mediated by type IV fimbriae called bundle-forming pili (4). Subsequently there is an alteration of the host cytoskeletal structure, including effacement of the surface microvilli, followed by the appearance of a pedestal-like structure upon which external bacteria are localized (2, 3, 5). Intimate association with epithelial cells requires a bacterial outer membrane protein, intimin, encoded by *eaeA* (6, 7). Host cell responses following contact with EPEC include induction of inositol trisphosphate and Ca^{2+} fluxes (8-10), protein phosphorylation (11, 12), and rearrangement of host cytoskeletal components under adherent bacteria (5, 13). Several bacterial mutants have been isolated which are defective in pedestal formation. Intimin is required for reorganizing the host cytoskeleton, although it is not needed for signal transduction (12). Mutations in other loci (*eaeB*, *cfm*, and class 5) fail to induce inositol phosphate fluxes, protein-tyrosine phosphorylation, and cytoskeletal rearrangements (9, 10, 12, 14, 15). However, the mechanisms by which products of these bacterial loci effect signal transduction in epithelial cells remained uncharacterized. To further elucidate these mechanisms, we examined whether EPEC produced proteins that interacted

with host epithelial cells. Indeed, we identified five distinct proteins that EPEC secretes that associate with HeLa cells, and here we show that secretion of these proteins is essential for mediating epithelial cell signal transduction. We also show that one of the secreted proteins is EaeB, and other mutants that are defective for triggering signal transduction are also defective for secretion of EaeB and three other proteins.

MATERIALS AND METHODS

Bacterial Strains. EPEC strains E2348/69, JPN15, JPN15/pCVD450, *eaeB* (UMD864), *eaeA* [10-5-1(1)], *cfm* [14-2-1(1) and 27-3-2(1)], class 5 [30-5-1(3)], and *bfpA* [31-6-1(1)] are described elsewhere (6, 14, 16, 17). They were grown in Luria-Bertani (LB) broth at 37°C without shaking.

HeLa Cells. HeLa cells (ATCC CCL2) were grown at 37°C, 5% CO_2 /95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum.

Secreted Proteins in Tissue Culture Medium. DMEM was inoculated with the bacterial culture to an OD_{600} of ≈ 0.01 and incubated overnight without shaking at either 30°C or 37°C. The bacteria were removed by centrifugation ($18,000 \times g$, 10 min) and the supernatant proteins were precipitated by addition of trichloroacetic acid [TCA, 10% (wt/vol)]. The proteins were pelleted after incubation on ice for 60 min and suspended in SDS/PAGE loading buffer before resolution by SDS/12% PAGE (18).

Preferential Labeling of Bacterial Proteins During Infection of HeLa Cells. Prior to infection, plates of confluent HeLa cells ($\approx 10^6$ cells) were preincubated for 40 min in cysteine- and methionine-free DMEM containing cycloheximide (100 μg /ml) and cytochalasin D (1 μg /ml). EPEC was added (multiplicity of infection, 100) and 200 μCi (7400 kBq) of [^{35}S]methionine/cysteine (Tran ^{35}S -label; ICN) was added at various times (see text). The supernatants were cleared by centrifugation in an Eppendorf centrifuge (13,000 rpm, 5 min, 4°C) and concentrated by TCA precipitation as above. The HeLa cells were washed two to three times in cold phosphate-buffered saline (PBS), lysed by the addition of 1% (vol/vol) Triton X-100 in the presence of protease inhibitors (12), and the soluble fraction was isolated. Equal amounts of radioactivity of the supernatant and the Triton-soluble fractions were subjected to SDS/12% PAGE and the separated proteins were detected by autoradiography.

Protease Protection Studies. HeLa monolayers were infected with wild-type or *eaeB* mutant EPEC strains for 150 min prior to preferential labeling of bacterial proteins as described above. After 30 min of radioactive labeling, the monolayers

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TCA, trichloroacetic acid.

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were washed three times with cold PBS. The Triton X-100-soluble fraction was isolated from one plate (see above) while an identical plate was incubated with proteinase K (50 μ g/ml) in PBS for 15 min on ice. The proteinase K-detached monolayer was isolated, pelleted, and washed with PBS to remove the protease. The host cell pellet was then extracted with lysis buffer containing 1% Triton X-100. The soluble fraction was isolated and TCA precipitated to stop any residual protease action. The secreted proteins were also exposed to proteinase K (50 μ g/ml) as above in the absence of host cells before being TCA precipitated. Proteins were again separated by SDS/12% PAGE and visualized by autoradiography.

Data Imaging. Autoradiographic and Coomassie-stained profiles were scanned (Agfa Studio Scanner) into Adobe Photoshop, where they were labeled before printing with a Mitsubishi S3600-30U color printer.

RESULTS

Secretion of Proteins by EPEC. We infected cultured epithelial cells with EPEC under conditions such that bacterial proteins were preferentially labeled. Thus, HeLa monolayers were infected with EPEC in the presence of cytochalasin D (12) (to block bacterial uptake) and cycloheximide (19) (to inhibit host protein synthesis), and [35 S]methionine/cysteine was added for 30 minutes at half-hour intervals over a 3-hr period. After each time interval, the medium was collected, nonadherent bacteria were removed by centrifugation, and the supernatant was concentrated by TCA precipitation. The host monolayer was also washed with PBS and analyzed for associated bacterial proteins (see below). Analysis of the supernatant samples by SDS/PAGE and autoradiography demonstrated that EPEC specifically secreted five polypeptides of ca. 110, 40, 39, 37, and 25 kDa (Fig. 1). (The triplet of proteins at 37–40 kDa was difficult to resolve, but additional data indicating that it consists of three proteins are provided in Figs. 2–5.) The secretion of these proteins was not immediate, with a delay of 60–90 min before the appearance of the 110-kDa protein, with the others appearing after another 30 min. This lag period approximated those previously reported for EPEC

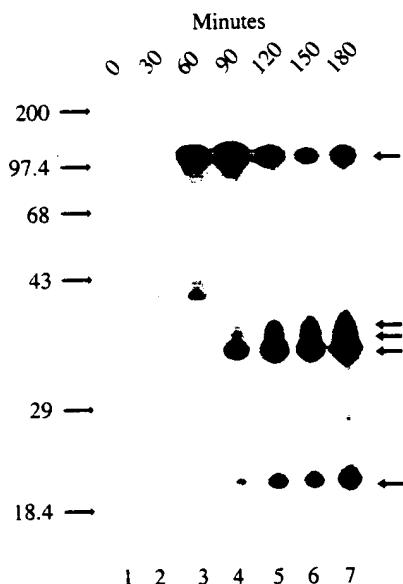


FIG. 1. Profile of proteins secreted into tissue culture medium by wild-type EPEC. Bacterial proteins were preferentially labeled in DMEM at 30-min intervals over the course of 3 hr. Bacteria were removed by centrifugation and supernatant samples were TCA precipitated and analyzed by SDS/PAGE prior to autoradiography. Positions of molecular size (kDa) markers are indicated at left. Arrows at right indicate the five secreted proteins.

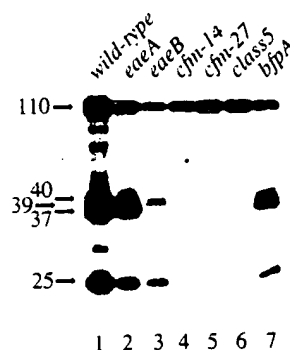


FIG. 2. Profile of secreted proteins of wild-type and various EPEC mutant strains in the presence of HeLa cells. Bacterial proteins were preferentially labeled for 30 min at time $t = 150$ min after initiation of infection of HeLa cells (with cytochalasin D and cycloheximide present). Supernatants were isolated and proteins were precipitated, resolved by SDS/12% PAGE, and visualized by autoradiography.

preceding attachment and invasion of HeLa cells and phosphorylation of the epithelial Hp90 protein (9, 12), suggesting that these protein may be involved in one or more of these processes. EPEC secretion of these proteins also occurred in tissue culture medium in the absence of epithelial cells, though growth in LB broth or M9 minimal medium did not induce secretion (data not shown).

EPEC Secreted Proteins: A Role in Signal Transduction?

Examination of several EPEC mutants defective in signal transduction (*eaeB*, two different *cfm* strains, and class 5) revealed that they were all defective in the secretion of at least one protein (Fig. 2). Whereas the *eaeB* mutant secreted all but the 37-kDa polypeptide (Fig. 2, lane 3), the two *cfm* mutants (lanes 4 and 5) and the class 5 mutant (lane 6) secreted only the 110-kDa polypeptide. In contrast, mutants which are not defective in signal transduction but are unable to focus host cytoskeletal components (*eaeA*; lane 2) or adhere poorly to the host cell (*bfpA*; lane 7) secreted all five polypeptides. No mutants were found that abolished the secretion of the 110-kDa protein.

Regulation of Secreted Products. Earlier studies revealed that EPEC incubated at 30°C did not activate host tyrosine phosphorylation or invade cultured cells (I. Rosenshine and B.B.F., unpublished observations). Supporting a role for secretion in the above processes, we found that, in contrast to bacterial growth at 37°C, growth at 30°C inhibited the secre-

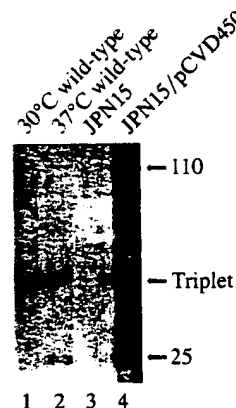


FIG. 3. Regulation of EPEC secretion by temperature and PerA. DMEM was inoculated with the indicated EPEC strains and grown at either 30°C (lane 1) or 37°C (lanes 2–4) overnight. The supernatant, derived from equal optical densities of bacteria, was TCA precipitated and resolved by SDS/PAGE prior to visualization by Coomassie blue staining.

Mass, kDa	Protein	1	5	10	15	20	23
110		A G L N I D N V W A R D Y L D L A Q N K G					
40		T ? K R N K D A A G ? D G ? Y P					
39	GAPDH	A E K V G I N G F G R I G R I V					
37	EaeB	M N T I D N N N A A I A V N S V L S S T T					
25		M D T S T T A S V A S A N A S T S T S M A Y N					

FIG. 4. Amino-terminal sequence data. Boldface letters indicate differences with *E. coli* cytoplasmic GAPDH protein (GapA).

tion of all the EPEC proteins in tissue culture medium, including the 110-kDa protein (Fig. 3).

Several EPEC virulence genes, including *eaeA* and *bfpA*, are under the control of a positive regulator encoded by the *perA* locus located on the virulence plasmid (refs. 17 and 20; J. Kaper, personal communication). A plasmid-cured strain of EPEC (JPN15, which is thus missing *perA* and *bfpA*) was transformed with a multicopy plasmid carrying the *perA* locus (pCVD450), and the supernatant was examined for the secreted proteins. Whereas the JPN15 strain secreted very low levels of the EPEC secreted proteins, the isogenic strain containing the cloned *perA* locus showed dramatically increased levels of protein secretion (Fig. 3). Thus, the *perA* virulence regulator locus and temperature appear to regulate directly or indirectly the production and/or secretion of these proteins.

EaeB Is One of the Secreted Proteins. The secreted proteins were isolated from supernatants derived from wild-type or *eaeB* EPEC, separated by SDS/PAGE, and transferred to poly(vinylidene difluoride) paper prior to amino-terminal sequence analysis (Fig. 4). The amino-terminal sequence of the 37-kDa polypeptide (21 amino acids) was identical to the predicted protein sequence encoded by the *eaeB* gene (15). This secreted protein was absent from the supernatant of the *eaeB* mutant (Fig. 2, lane 3) but both its secretion (data not shown) and triggering of signal transduction (10, 15) could be restored by the addition of the plasmid pMSD3, which carries the *eaeB* gene.

Fourteen out of 16 residues of the derived amino-terminal sequence of the 39-kDa product matched that of the *E. coli* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with the two mismatches occurring at positions 1 and 2 (Fig. 4). None of the 110-, 40-, or 25-kDa protein sequences shared significant homology with sequences in the data bases (21).

Association of the Secreted Proteins with Epithelial Cells. We next examined whether the EPEC secreted proteins interacted with HeLa cells. After infection with EPEC (in the

presence of cytochalasin D and cycloheximide) and radiolabeling, the supernatant was removed and the HeLa cell monolayer was extensively washed. The cells were then extracted with 1% Triton X-100 and divided into a Triton-insoluble fraction (containing cytoskeletal components, nuclei, and adherent radiolabeled bacteria) and a Triton-soluble fraction (containing epithelial cytoplasmic and membrane proteins) (12). Analysis of the Triton-soluble fraction by SDS/PAGE and autoradiography demonstrated that only five radiolabeled bacterial proteins, corresponding in size to the secreted proteins, remained associated with HeLa cells after washing (Fig. 5A). Extraction with Triton X-100 of radiolabeled and washed EPEC collected from the monolayer supernatants did not yield any of the five secreted proteins (Fig. 5B). We also examined secreted protein association with HeLa cells, using various EPEC mutants. We found that with the secretion-defective mutants (*cfm* and class 5 mutants), at least EaeB and the 25-kDa proteins did not associate with HeLa cells (Fig. 5C, lanes 4–6), indicating that secretion is needed for this association.

Protease Resistance of EaeB After Association with HeLa Cells. The above results led us to speculate that the secreted molecules may interact intimately with the host cell to transduce signals and thus may become resistant to externally added proteases. Therefore, we compared the protease sensitivity properties of the radiolabeled EPEC secreted proteins with that of the same molecules when associated with the host monolayer (Fig. 6). Although addition of protease to the cell-free secreted proteins rapidly degraded all but the 110-kDa protein (Fig. 6, lanes 5–8), EaeB (the 37-kDa protein) became resistant to this treatment when associated with the host cell. This result suggests that EaeB, an essential signal transduction molecule, may adopt a different conformation upon association with the host cell, possibly due to it becoming embedded in the membrane or perhaps gaining access to an internal epithelial compartment.

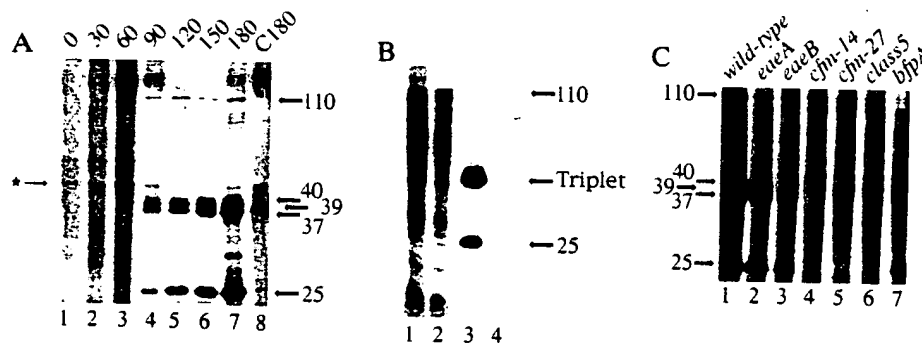


FIG. 5. Triton extraction of infected HeLa monolayers and radiolabeled EPEC. (A and C) HeLa cells were infected with wild-type (A) or mutant (C) EPEC strains and bacterial proteins were preferentially labeled in the presence of cytochalasin D and cycloheximide for 30-min intervals over the course of a 3 hr infection (A) or for 30 min following 150 min of infection (C). The unattached bacteria and supernatant were removed and the monolayer was washed thrice in cold PBS. The Triton X-100-soluble fraction was extracted. Proteins were resolved by SDS/PAGE and visualized by autoradiography. In A, C180 (lane 8) corresponds to an uninfected HeLa control in which radiolabel was added at 180 min for 30 min. The star marks a mammalian protein which was labeled in the presence of cycloheximide. (B) Radiolabeled profile of EPEC before (lane 1) and after (lane 2) Triton X-100 extraction, the EPEC proteins that were extracted by Triton X-100 (lane 4), and the proteins secreted by EPEC (lane 3).

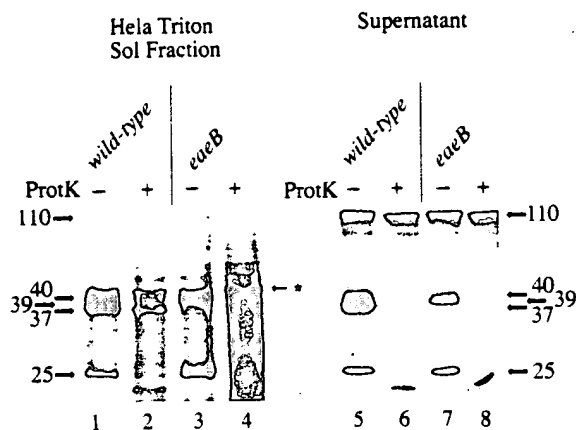


FIG. 6. Protease resistance of EaeB (37-kDa polypeptide) upon association with epithelial monolayers. HeLa cells were infected with either wild-type or *eaeB* EPEC strains for 150 min in the presence of cytochalasin D and cycloheximide. Radiolabel was added for 30 min, the supernatant was removed, and the monolayer was washed thrice in cold PBS. The HeLa Triton-soluble fraction was isolated from the control plate and a second plate was incubated on ice for 15 min with 50 μ g of proteinase K (ProtK) in 1 ml of PBS containing cycloheximide at 100 μ g/ml. The detached host cells were pelleted and washed with PBS to remove the proteinase K. The host cells were pelleted and lysed by the addition of 1% Triton X-100. The Triton-soluble fraction was isolated and TCA precipitated to stop any residual protease activity (Left). Cell-free supernatant proteins were also exposed to proteinase K (50 μ g/ml) for 15 min on ice before being TCA precipitated (Right). Proteins were resolved by SDS/PAGE and visualized by autoradiography. The star highlights a mammalian protein which was labeled in the presence of cycloheximide.

DISCUSSION

We have identified five proteins that EPEC secretes into the culture supernatant when grown in tissue culture medium (in the presence or absence of epithelial cells). Although not previously documented for EPEC, secretion of virulence factors is a common theme among pathogens (22). Indeed, EPEC secretion is not immediate upon infection but appears to require a substantial lag period which may reflect the requirement to adapt to the minimal conditions in the tissue culture fluid after prior growth in rich LB broth, a medium which does not permit protein secretion. A similar regulation has been suggested to explain a comparable lag in EPEC adherence (23). Intriguingly, in the presence of epithelial cells these same proteins appear to associate with the mammalian cells. Indeed, protease studies support the hypothesis that one of these proteins, EaeB, which is an essential signal transduction molecule, becomes resistant to external protease digestion upon association with the host cell. Unfortunately, attempts to complement signal transduction defects of EPEC mutants by the addition of bacteria-free supernatants were unsuccessful. It is possible that bacterial adherence is required for efficient interaction of the secreted molecules with the host cell and that secretion near the host cell surface may provide localized concentrated delivery of such molecules.

A role for these secreted proteins in signal transduction is supported by several lines of evidence. Comparison of the secretion profiles of EPEC mutants proficient in signal transduction of HeLa cells but defective in other aspects of epithelial cell interaction [such as binding (*bfpA*) or focusing host cytoskeletal products (*eaeA*)] with signal transduction-defective mutants (*eaeB*, *cfm*, and class 5) revealed several differences. Whereas the *bfpA* and *eaeA* mutants secreted all five proteins, the signal transduction mutants were missing a single 37-kDa secreted protein (*eaeB*) or four secreted proteins (*cfm* and class 5), signifying a phenotypic difference between the *eaeB* and the *cfm*/class 5 mutants, which are all defective

for triggering signals in epithelial cells (9, 10, 12, 14, 15). Amino-terminal sequence analysis identified the secreted 37-kDa protein, which is absent from the *eaeB* mutant, as the predicted *eaeB* gene product (which has a predicted molecular mass of 39 kDa). This polypeptide contains no conventional signal sequence and its amino terminus is not processed. Expression of cloned EaeB in the *eaeB* mutant results in signaling (10, 15) and restores the secretion of the 37-kDa protein (data not shown), supporting a critical role for this protein in signal transduction.

Although EaeB undoubtedly plays an important role in signal transduction, we can only hypothesize about the roles of the other secreted proteins and their role in virulence. However, like EaeB, the 40-, 39-, and 25-kDa proteins appear to share the same secretion pathway (see below), as both *cfm* and class 5 mutants are defective in secretion of all these proteins. Additionally, these proteins and the 110-kDa protein are all regulated by conditions known to regulate other known EPEC virulence factors such as intimin and bundle forming pili.

Comparison of the derived amino-terminal sequences of the secreted proteins with protein data bases revealed no significant homologies for the 110-, 40-, or 25-kDa protein. Intriguingly, the amino-terminal sequence of the 25-kDa protein matches an open reading frame located between *eaeA* and *eaeB* (B.K. L.-C. Lai, B.B.F. and M. Donnenberg, unpublished data), a region which is necessary for EPEC pathogenesis. The amino-terminus of the 39-kDa polypeptide is highly homologous to that of GAPDH, a cytoplasmic protein involved in the glycolytic pathway. The apparent secretion of this protein is unusual, although Pancholi and Fischetti (24) have reported that a GAPDH-like protein is exported to the outer membrane of streptococci and can bind host cell surface components. More recently, Pancholi and Fischetti (25) have shown that this molecule can become autoribosylated, a mechanism used in higher organisms to modify protein function.

Both the *cfm* and class 5 mutants are defective for secretion of the 40-kDa, 39-kDa, 37-kDa (EaeB), and 25-kDa proteins, suggesting that these mutants contain defects in an export pathway. Indeed, the two *cfm* mutants carry different insertions in a multigenic export operon similar to that found in other pathogens (26–29), while the class 5 insertion remains uncharacterized (14, 37). This novel export pathway has been called a type III secretion pathway and apparently does not rely on classical cleavable signal sequences (as is the case for EaeB and the 25-kDa protein). A homologous pathway directs the secretion of *Salmonella* and *Shigella* virulence proteins which are essential for bacterial invasion (28, 30), although their mechanisms of action are still unknown. A similar system in *Yersinia* mediates secretion of several virulence proteins such as YopH, which has an antiphagocytic activity (31), and YopE, which is injected into the host cell upon bacterial contact, leading to actin microfilament depolymerization and cytotoxicity (32). It is not clear whether, like the *Yersinia* YopE protein, one or more of the EPEC secreted proteins are injected directly into the host cytoplasm or possibly transferred to a host membrane location where they function to induce host signaling pathways. However, the observation that a central signal transduction molecule, EaeB (37 kDa), becomes resistant to externally added protease upon association with the host cell introduces the intriguing possibility that, like YopE, EaeB may gain access to an internal location where it alters host signal transduction pathways.

Bacterial virulence gene expression is normally tightly regulated and is influenced by many factors including temperature, osmolarity, stress, and nutrient availability (33–36). Indeed, protein secretion of virulence factors in *Shigella*, *Yersinia*, and EPEC is negatively regulated by low temperature. In the case of *Shigella*, expression is regulated by VirR, apparently via DNA supercoiling (35). Another positive regulator, VirF, which itself appears to be thermoregulated, is also required for

Shigella virulence expression (33). Like *Shigella*, the secretion of EPEC secreted proteins is thermoregulated and under the control of a positive regulator, PerA.

We analyzed several bacterial strains related to EPEC, including a rabbit-specific EPEC (RDEC-1) and an enterohemorrhagic *E. coli* (EHEC) serotype to determine whether these other pathogens also secrete similar proteins. All of these strains secreted proteins that were similar in molecular weight to the EPEC secreted proteins, although there was minor variation in the apparent molecular weights between strains (data not shown). Given that these pathogens and others such as *Citrobacter freundii* and *Hafnia alvei* cause similar cytoskeletal rearrangements leading to attaching/effacing lesions, it is possible that these organisms utilize similar secreted proteins to effect disease. Nonpathogenic laboratory strains of *E. coli* do not secrete similar proteins.

This paper reports the secretion of proteins by EPEC and reveals an essential role for secretion in transducing signals to the host cell. Characterization of these secreted proteins identified one as the product of *eaeB*, a gene previously known to be essential for signal transduction, while the others appear to be novel. In addition, we found several features of this system that are common to other bacterial pathogens. These include protein secretion via a type III secretion pathway, positive and negative regulation of virulence factors, and association of the secreted protein(s) with the host cell. Although there is an undeniable role for the secreted EaeB protein in signal transduction, roles for the other four secreted proteins can, as yet, only be inferred.

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